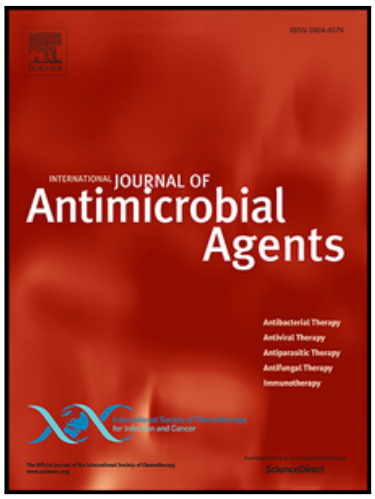

Antimicrobial susceptibility of Clostridium difficile isolated from food and environmental sources in Western Australia

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Highlights

- Antimicrobial resistance was observed in *C. difficile* from food, compost and lawn.
- Compost isolates were more often resistant to erythromycin and tetracycline.
- Multidrug resistance was detected in four strains, all isolated from compost.
- Similar resistance patterns were noted in environmental and human *C. difficile*.
- Environmental *C. difficile* could be a reservoir for CA-CDI.

Title: Antimicrobial susceptibility of *Clostridium difficile* isolated from food and environmental sources in Western Australia

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ABSTRACT (250 words)

We recently reported a high prevalence of *Clostridium difficile* in retail vegetables, compost and lawn in Western Australia (WA). The objective of this study was to investigate the antimicrobial susceptibility of previously isolated food and environmental *C. difficile* isolates of WA. A total of 274 *C. difficile* isolates from vegetables, compost and lawn were tested for susceptibility to a panel of 10 antimicrobial agents (fidaxomicin, vancomycin, metronidazole, rifaximin, clindamycin, erythromycin, amoxicillin/clavulanate, moxifloxacin, meropenem and tetracycline) using an agar incorporation method. Fidaxomicin was the most potent agent (MIC_{50}/MIC_{90}, 0.06/0.12 mg/L). Resistance to fidaxomicin and metronidazole was not detected, and resistance against vancomycin (0.7%) and moxifloxacin (0.7%) was low. However, 37.6% of isolates showed resistance to at least one agent and multidrug resistance was observed in 3.9% of the resistant isolates, all of which came from compost. A significantly greater proportion of compost isolates were resistant to clindamycin, erythromycin and tetracycline compared to food and/or lawn isolates. *C. difficile* RT 014/020 showed more clindamycin resistance than other less common RTs (Chi-square $p = 0.008$). Contaminated vegetables, compost and lawn could be playing an intermediary role in the transmission of *C. difficile* from animals to humans. Environmental strains of *C. difficile* could also function as a reservoir for antimicrobial resistance genes of clinical relevance. This study provides a baseline for future surveillance of antimicrobial resistance in environmental *C. difficile* in Australia. (Word count = 228)

Keywords: *Clostridium difficile*, antimicrobial, resistance, food, environmental, Australia

1. Introduction

Clostridium difficile infection (CDI) is the leading cause of life-threatening infectious diarrhoea in humans, and a major public health issue in many developed countries [1]. *C. difficile* causes a wide range of symptoms, from mild diarrhoea to severe pseudomembranous colitis and, in rare cases, fulminant colitis that may lead to intestinal perforation or megacolon and sepsis [1]. The major risk factor for developing CDI is exposure to antimicrobials, particularly agents with activity against commensal bowel flora such as clindamycin, aminopenicillins, extended-spectrum cephalosporins and fluoroquinolones [1]. Since 2000, a substantial increase in the incidence of CDI has been observed worldwide, including community-associated CDI (CA-CDI). Currently, approximately 30% of all CDI cases in Australia are CA-CDI with no traditional risk factors such as hospital stay, previous antimicrobial use or old age/immune senescence [2].

Some studies have reported genetically-related, and in some cases indistinguishable, *C. difficile* strains from animals and humans [3] suggestive of zoonotic transmission. However, most related isolates were separated by vast geographical distances and there was no known prior contact between hosts, making direct transmission unlikely. Thus, following the isolation of clinically important *C. difficile* strains from food and the environment, it has been hypothesised that some CA-CDI might be foodborne or that transmission from an environmental source occurs in some other way [4].

Recently, we found a high prevalence of *C. difficile* on retail root vegetables (30.0%, 30/100) [5], in compost (27.2%, 22/81) destined for use in farming and landscaping (S.C. Lim *et al*., unpublished), and in public lawns (58.5%, 182/311) [6] in Perth, Western Australia (WA). It is possible that these contaminated items could be playing a role in the

transmission of CDI, especially CA-CDI. It is common practice in Australia to use composted animal manure as fertiliser for vegetable and lawn (turf) farming which could result in contamination of vegetables and lawn with *C. difficile* of animal origin and lead to genetically highly-related human CDI in the community.

While human clinical isolates are occasionally surveyed for antimicrobial susceptibility, less is known about antimicrobial resistance in *C. difficile* from other sources. The aims of this study were to (i) determine the antimicrobial susceptibility of *C. difficile* isolated from food, compost and lawn to a panel of 10 antimicrobial agents, and (ii) compare the antimicrobial profile of these strains with published animal- and human-derived isolates from Australia.

2. Materials and methods

2.1. Bacterial isolates

In 2015 and 2016, studies were carried out in WA to determine the prevalence of *C. difficile* in food and environmental sources. All *C. difficile* isolates from those studies (*n* = 274) were tested in the current investigation: 56 from vegetables (43 from root vegetables [5] and 13 from imported vegetables and/or vegetables of unknown country of origin (unpublished data)), 36 from compost (unpublished data) and 182 from lawn [6]. The isolates had been characterised by toxin gene profiling and PCR ribotyping [5, 6] using a reference library consisting of a collection of 54 internationally recognised UK ribotypes (RTs) that included 15 reference strains from the European Centre for Disease Prevention and Control, and various RTs currently circulating in Australia assigned with internal nomenclature, prefixed with QX. A summary of the RTs, toxin gene profiles and sources of the 274 *C. difficile* isolates is shown in Fig. 1. *C. difficile* RT 014 and 020 were grouped together due to nearly identical RT banding patterns differing only by one band. *C. difficile* RT 014/020,

which is the most common RT isolated from Australian pigs and humans [3], was the most common food and environmental RT in the collection. The toxin profiles represented included A-B-CDT- (*n* = 145; 52.9%), A+B+CDT- (*n* = 117; 42.7%), A-B+CDT+ (*n* = 7; 2.6%), A+B+CDT+ (*n* = 2; 0.7%), A-B-CDT+ (*n* = 2; 0.7%) and A-B+CDT- (*n* = 1; 0.4%).

2.2. MIC determination by agar incorporation

The minimum inhibitory concentrations (MIC) of a panel of 10 antimicrobial agents were determined by the agar incorporation method as described by the CLSI [7, 8]. The panel comprised first line CDI therapies vancomycin and metronidazole, as well as fidaxomicin, rifaximin, clindamycin, erythromycin, amoxicillin/clavulanate, moxifloxacin, meropenem and tetracycline. The clinical breakpoints for vancomycin and metronidazole were those recommended by EUCAST (http://eucast.org). For fidaxomicin, the European Medical Agency proposed breakpoint of 1 mg/L was used (report WC500119707, http://www.ema.europa.eu/). Rifaximin resistance (≥ 32 mg/L) was as described by O'Connor *et al.* [9] and the breakpoints for clindamycin, erythromycin, amoxicillin/clavulanate, moxifloxacin, meropenem and tetracycline were those provided by CLSI [8].

2.3. Statistical analysis

Kruskal-Wallis rank sum test and Dunn test were performed to compare the geometric mean of MICs between *C. difficile* of food, compost and lawn origins. Fisher's exact test, Chi-square test and post hoc test were used to compare the resistance rates of *C. difficile* from different origins.

3. Results

Fidaxomicin was the most active agent, showing potent *in vitro* activity against all isolates (MIC_{50}/MIC_{90}, 0.06/0.12 mg/L; MIC range < 0.002 – 0.5 mg/L) (Table 1). This

activity was superior to the recommended first-line treatment agents for CDI, vancomycin (MIC_{50}/MIC_{90}, 1/2 mg/L) and metronidazole (MIC_{50}/MIC_{90}, 0.25/0.5 mg/L). However, no metronidazole resistance was observed and only two (0.73%) isolates were resistant to vancomycin (MIC = 4 mg/L, resistant breakpoint > 2).

Phenotypic resistance to at least one antimicrobial agent was observed in 103 (37.6%) of the 274 isolates, predominantly those from compost (14/36, 38.9%) and lawn (82/182, 45.1%). Only 7/56 food isolates (12.5%) exhibited resistance. Multidrug resistance (MDR), defined as resistance to at least one agent in three or more antimicrobial categories, was observed in 4 (3.9%) of the 103 resistant isolates and all were of compost origin; one isolate each of QX 327 (A-B-CDT-), QX 140 (A-B-CDT-) and UK 046 (A+B+CDT-) were resistant to clindamycin (MIC = > 32 mg/L), erythromycin (MIC = > 256 mg/L) and tetracycline (MIC = 64 mg/L, 16 mg/L and 32 mg/L, respectively), and one isolate of UK 012 (A+B+CDT-) was resistant to rifaximin (MIC = > 64 mg/L), erythromycin (MIC = > 256 mg/L) and tetracycline (MIC = 64 mg/L). Almost one-third (4/14) of the resistant compost isolates were MDR.

There was a significant association between the source of isolates and resistance to rifaximin (3.6% from food, 2.8% from compost, 0.0% from lawn; Fisher's exact $p = 0.037$), clindamycin (7.1% from food, 30.6% from compost, 42.3% from lawn; Chi-square $p = < 0.0001$), erythromycin (0.0% from food, 19.4% from compost, 1.1% from lawn; Fisher's exact $p = < 0.0001$) and tetracycline (1.8% from food, 13.9% from compost and 1.1% from lawn; Fisher's exact $p = 0.002$) (Table 1). Compared to food and lawn isolates, compost isolates were more often resistant to erythromycin (compost vs. food, post hoc test $p = 0.001$; compost vs. lawn, post hoc test $p = 0.0002$) and tetracycline (compost vs. food, post hoc test $p = 0.049$; compost vs. lawn, post hoc test $p = 0.005$) (Table 1). The proportion of lawn isolates resistant to clindamycin was similar to that of compost isolates (post hoc test $p =$

0.26); however, both were significantly higher than food isolates (food vs. compost, post hoc test $p = 0.01$; food vs. lawn, post hoc test $p = < 0.0001$). Susceptibility to fidaxomicin, vancomycin, metronidazole, amoxicillin/clavulanate, moxifloxacin and meropenem did not significantly vary between isolates from different sources.

4. Discussion

While there has been an increase in publications on the prevalence of *C. difficile* in food and the environment, few reports have investigated the antimicrobial susceptibility of isolates of *C. difficile* recovered [10, 11]. This study is the first to determine the antimicrobial resistance patterns of food and environmental *C. difficile* isolates in Australia.

As macrolides and tetracycline-based antimicrobial agents constitute approximately 40% (112.2 tonnes per year) of all antimicrobials used in Australian food animals [12], it was not surprising that *C. difficile* isolates from compost exhibit resistance to erythromycin and tetracycline. Although clindamycin is not approved for use in food animals, previous studies on animal *C. difficile* strains have frequently reported intermediate or resistant MICs of clindamycin [3, 13]. Our previous study on *C. difficile* RT 014 from pigs showed high (69%) non-susceptibility to clindamycin, erythromycin and tetracycline, and 100% susceptibility to fidaxomicin, vancomycin, metronidazole, rifaximin, amoxicillin/clavulanate, moxifloxacin and meropenem [3], in agreement with the resistance patterns of compost isolates in this study. These findings, taken together, support our theory that compost isolates are likely to be of animal origin as *C. difficile* spores will survive the composting process [14]. Although not indicative of transmission to humans, both compost and lawn isolates shared antimicrobial resistance/susceptibility patterns similar to those reported in human-derived isolates [15]. In 2015, antimicrobial susceptibility testing was performed on 440 human *C. difficile* isolates collected across five Australian states [15]. In that study, the majority of isolates were

susceptible to fidaxomicin (100%), vancomycin (100%), metronidazole (100%), rifaximin (100%), amoxicillin/clavulanate (100%), moxifloxacin (96.1%) and meropenem (99.5%); similar to the findings for food, compost and lawn isolates. Furthermore, *C. difficile* that causes human CDI in Australia has a high prevalence of clindamycin resistance (84.3%) [15]. In this study, both compost and lawn isolates had relatively high levels of clindamycin resistance with MICs comparable to isolates from humans (MIC_{50}/MIC_{90}, 8/> 32 mg/L; MIC range 0.5 – > 32 mg/L) [15]. Further studies using whole-genome sequencing are necessary to better determine the relatedness of these compost and lawn isolates with *C. difficile* isolated from humans, particularly in the community.

C. difficile RT 014/020 is consistently one of the most frequently isolated toxigenic RTs in humans worldwide, including Australia [16-18]. Of public health interest, in this study, *C. difficile* RT 014/020 (35/78, 44.9%) exhibit significantly greater resistance to clindamycin, a reported risk factor for CA-CDI [19], compared to other less common RTs (54/196, 27.6%) (Chi-square $p = 0.008$). Furthermore, two of the four MDR isolates, RTs 012 and 046 from compost, were toxigenic and RTs that have previously been associated with human CDI [17]. This suggests that environmental *C. difficile* could be a reservoir for antimicrobial resistance genes of clinical relevance. In addition, exposure to toxigenic and antimicrobial resistant food and environmental *C. difficile* poses a risk of infection to susceptible individual and re-infection of a resolved patient with a new strain of *C. difficile* while the gut microbiota is still compromised.

Based on antimicrobial resistance patterns, the *C. difficile* strains from compost appeared quite different to strains from vegetables and lawn; with greater resistance to erythromycin, tetracycline and/or clindamycin. This may be a reflection of a sampling bias as it is possible that the compost used to fertilise the vegetables and lawn came from different farms with different antimicrobial usage. It was impossible to determine which animal farm

the manure originally came from due to issues of confidentiality. Furthermore, the acquisition and loss of antibiotic resistance genes occurs readily in *C. difficile* in response to selective pressure, as *C. difficile* has a diverse and highly flexible accessory genome comprising a range of mobile genetic elements conferring resistance to macrolide/lincosamide [Tn*6194*/Tn*5398* (*ermB*)] and tetracycline [Tn*916*/Tn*5397* (*tetM*)], many of which are capable of inter- and intra-species transfer *in vitro* [1, 3, 20].

In summary, similarities in antimicrobial resistance/susceptibility patterns were observed between environmental *C. difficile* and those of animal and human origin. Future genomic studies are required to determine if these isolates do indeed originate from animals and are responsible for CA-CDI. Nevertheless, this study shows that food or the environment harbouring toxigenic *C. difficile* strains could be sources for CDI in the community. This study provides a baseline for future surveillance of antimicrobial resistance in food and environmental *C. difficile* in Australia.

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Declarations

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Competing Interests: None declared.

Ethical Approval: None required.

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Fig. 1. Origin of *C. difficile* RTs tested in this study (*n* = 274). RT others (*) QX 001 (A+B+CDT-), QX 026 (A+B+CDT-), QX 067 (A-B-CDT-), QX 076 (A+B+CDT-), QX 121 (A-B-CDT-), QX 122 (A-B-CDT-), QX 140 (A-B-CDT-), QX 274 (A+B+CDT+), QX 327 (A-B-CDT-), QX 399 (A+B+CDT+), QX 409 (A+B+CDT-), QX 449 (A-B-CDT-), QX 463 (A-B-CDT-), QX 519 (A+B+CDT-), QX 525 (A-B-CDT-), QX 546 (A-B-CDT-), QX 547 (A-B+CDT+), QX 550 (A-B-CDT-), QX 597 (A-B-CDT-), QX 598 (A-B-CDT-), QX 599 (A-B-CDT-), QX 600 (A-B-CDT+), QX 602 (A-B-CDT-), QX 603 (A-B-CDT-), QX 604 (A-B-CDT-), QX 605 (A-B-CDT-), QX 606 (A-B-CDT-), QX 607 (A-B-CDT-), UK 005 (A+B+CDT-), UK 009 (A-B-CDT-), UK 017 (A-B+CDT-), UK 018 (A+B+CDT-), UK 033 (A-B-CDT+), UK 046 (A+B+CDT-), UK 064 (A+B+CDT-), UK 070 (A+B+CDT-), UK 077 (A-B-CDT-), UK 080 (A-B+CDT+), UK 106 (A+B+CDT-), UK 137 (A+B+CDT-) and UK 584 (A-B+CDT+).

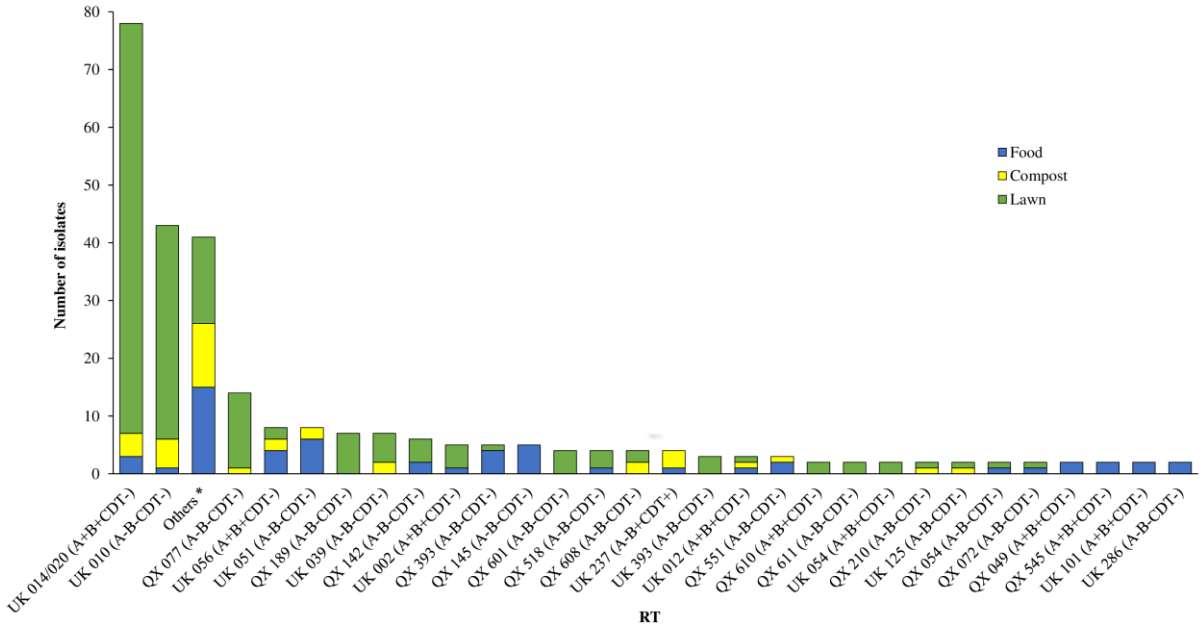

Table 1 Summary MIC data of 274 *C. difficile* isolates from food, compost and lawn

			Breakpoints			MIC			GM			
Agent	**Origin**	***N***	**S**	**I**	**R**	**range (mg/L)**	**MIC_{50} (mg/L)**	**MIC_{90} (mg/L)**	**(mg/L)**	**%R (*n*)**	**%NR (*n*)**	***p*-value[†]**
FDX[d]	Food	56	-	-	≥ 1	0.004 - 0.12	0.06	0.06	0.03[‡]	0 (0)	100 (56)	
	Compost	36				0.004 - 0.5	0.06	0.12	0.06	0 (0)	100 (36)	
	Lawn	182				< 0.002 - 0.12	0.06	1	0.06	0 (0)	100 (182)	
	Total	274				< 0.002 - 0.5	0.06	0.12	0.05	0 (0)	100 (274)	1
VAN[a]	Food	56	≤ 2	-	> 2	0.5 - 2	2	2	1.41	0 (0)	100 (56)	
	Compost	36				1 - 2	1	2	1.26	0 (0)	100 (36)	
	Lawn	182				0.25 - 4	1	2	1.27	1.1 (2)	98.9 (180)	
	Total	274				0.25 - 4	1	2	1.29	0.7 (2)	99.3 (272)	1
MTZ[a]	Food	56	≤ 2	-	> 2	0.25 - 1	0.5	0.5	0.40[§]	0 (0)	100 (56)	
	Compost	36				0.25 - 2	0.25	0.5	0.29	0 (0)	100 (36)	
	Lawn	182				0.12 - 1	0.25	0.5	0.30	0 (0)	100 (182)	
	Total	274				0.12 - 1	0.25	0.5	0.32	0 (0)	100 (274)	1
RFX[c]	Food	56	-	-	≥ 32	0.004 - 64	0.008	8	0.04	3.6 (2)	96.4 (54)	
	Compost	36				0.004 - > 64	0.008	0.25	0.02	2.8 (1)	97.2 (35)	
	Lawn	182				0.002 - 16	0.004	1	0.01[‡]	0 (0)	100 (182)	
	Total	274				0.002 - > 64	0.008	4	0.02	1.1 (3)	98.9 (271)	**0.037**
CLI[b]	Food	56	≤ 2	4	≥ 8	0.12 - 8	2	4	1.97[‡]	7.1 (4)	92.9 (52)	
	Compost	36				1 - > 32	4	> 32	4.49	30.6 (11)	69.4 (25)	
	Lawn	182				0.12 - > 32	4	16	3.71	42.3 (77)	57.7 (105)	
	Total	274				0.12 - > 32	4	16	3.34	33.6 (92)	66.4 (182)	< **0.0001**
ERY[b]	Food	56	-	-	> 8	0.25 - 2	1	1	0.74	0 (0)	100 (56)	
	Compost	36				0.5 - > 256	1	> 256	2.94[§]	19.4 (7)	80.6 (29)	
	Lawn	182				0.06 - > 256	1	2	0.81	1.1 (2)	98.9 (180)	
	Total	274				0.06 - > 256	1	2	0.94	3.3 (9)	96.7 (265)	< **0.0001**
AMC[b]	Food	56	≤ 4	8	≥ 16	0.125 - 1	0.5	0.5	0.41	0 (0)	100 (56)	
	Compost	36				0.06 - 4	0.25	1	0.44	0 (0)	100	

											(36)	
	Lawn	182				0.125 - 8			0.66[§]		100	
							0.5	1		0 (0)	(182)	
	Total	274				0.06 - 8			0.57		100	
							0.5	1		0 (0)	(274)	1
MXF[b]	Food	56	≤ 2	4	≥ 8	0.5 - 4			1.43		100	
							2	2		0 (0)	(56)	
	Compost	36				1 - 4			1.56		100	
							2	2		0 (0)	(36)	
	Lawn	182				0.25 - 8			1.40	1.1	98.9	
							2	2		(2)	(180)	
	Total	274				0.25 - 8			1.43	0.7	99.3	
							2	2		(2)	(272)	1
MEM[b]	Food	56	≤ 4	8	≥ 16	1 - 4			2.73		100	
							2	4		0 (0)	(56)	
	Compost	36				1 - > 16			3.30[¶]	5.6	94.4	
							4	4		(2)	(34)	
	Lawn	182				0.5 - 16			2.42	0.5	99.5	
							2	4		(1)	(181)	
	Total	274				0.5 - > 16			2.58	1.1	98.9	
							2	4		(3)	(271)	0.071
TET[b]	Food	56	≤ 4	8	≥ 16	0.06 - 64			0.15	1.8	98.2	
							0.12	0.25		(1)	(55)	
	Compost	36				0.06 - 64			0.49[§]	13.9	86.1	
							0.12	16		(5)	(31)	
	Lawn	182				0.03 - 16			0.17	1.1	98.1	
							0.12	0.5		(2)	(180)	
	Total	274				0.03 - 64			0.19	2.9	97.1	
							0.12	0.5		(8)	(266)	**0.002**

FDX, fidaxomicin; VAN, vancomycin; MTZ, metronidazole; RFX, rifaximin; CLI, clindamycin; ERY, erythromycin; AMC, amoxicillin/clavulanate; MXF, moxifloxacin; MEM, meropenem; TET, tetracycline; MIC, minimum inhibition concentration; S, susceptible; I, intermediate; R, resistance; NR, non-resistance; GM, geometric mean.

[a] Breakpoints are those recommended by EUCAST (http://eucast.org) based on the epidemiological cut-off values for the 'wild-type' population.

[b] Breakpoints are those recommended for anaerobes by CLSI.[11]

[c] Resistance (≥ 32 mg/L) is as described by O'Connor *et al.*[12]

[d] Resistance (≥ 1 mg/L) is recommended by EMA (report WC500119707, http://www.ema.europe.eu/).

[†] Chi-square / Fisher exact to compare *C. difficile* %R of food, compost and lawn origins

[‡] GM is significantly lower than the other two origins (FDX, $p < 0.0001$; RFX, $p < 0.005$; CLI, $p < 0.0001$)

[§] GM is significantly higher than the other two origins (MTZ, $p < 0.0001$; ERY, $p < 0.005$; AMC, $p < 0.0001$; TET, $p < 0.0001$)

[¶] GM is significantly higher than the lawn isolates, but not the food isolates (MEM, $p < 0.005$)